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SOME EFFECTS OF HYDROCORTISONE ON THE EARLY DEVELOPMENT OF THE RAT COTTON PELLET GRANULOMA

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Abstract—Differential cell counts, DNA levels, cellular proliferation (studied by *in vitro* [^{3}H]thymidine incorporation) and hydrolytic enzyme release into inflammatory exudates were investigated in sterile cotton wool pellets at various times after subcutaneous implantation in rats. At 2 days (the earliest time studied) the predominant cell type was the polymorphonuclear leucocyte (PMNL), but by 5 and 7 days increasing numbers of mononuclear cells (macrophages and lymphocytes) were present. Total DNA levels were almost constant, usually increasing slightly with time. DNA synthesis did not occur before day 3 and was still increasing at day 7. The two lysosomal hydrolases measured (*N*-acetyl-glucosaminidase) and β-glucuronidase) were present in the cell-free pellet exudate in high concentrations, suggesting that they had been released during phagocytosis. Treatment with hydrocortisone (10 mg/kg/day p.o.) during the earlier phase of the response reduced dry granuloma weight, total DNA levels and release of hydrolytic enzymes, and delayed the onset of cellular proliferation. Steroid treatment during the later proliferative phase only (days 3 to 6), reduced dry granuloma weight, total DNA level and hydrolytic enzyme release but did not affect [^{3}H]thymidine incorporation.

Since orally administered cortisone was first shown to inhibit the development of a rat cotton pellet granuloma [1], the effectiveness of other steroid anti-inflammatory agents in this test has been reported [2, 3]. The activities of non-steroidal anti-inflammatory drugs are less well-defined, different groups claiming that it is possible [4] and not possible [2, 5] to obtain dose-related inhibition of granuloma formation with these compounds, and a recent review [6] concludes that the test does not demonstrate satisfactorily the anti-inflammatory activities of non-steroidal compounds.

Although the test has been modified to give increased granuloma formation [7, 8] and hence a better response to non-steroidal anti-inflammatory drugs, the original test of Meier [1] appears to have the advantage that it provides a method of distinguishing "steroidal" type drugs from their non-steroidal counterparts.

The sequential physiological response to the implanted cotton pellet has been described by Swingle and Shideman [9] and can conveniently be divided into 3 phases; an initial increase in vascular permeability at the site of implantation, followed after 3 hr by an accumulation of exudate around the pellet and, finally, after 3 days the proliferation of fibroblasts and laying down of connective tissue, characterized by the appearance of collagen, around the pellet.

In view of the potential value of this test in identifying drugs capable of producing a "steroid-like" inhibition of granuloma formation, we have investigated the effect of hydrocortisone on several biochemical parameters at the site of pellet implantation. Our observations have been confined to the first 7 days of granuloma formation covering all three of the phases described [9]. In general we find that hydrocortisone, whilst active during the initial pellet response, is particularly effective during the later proliferative phase in reducing DNA levels, and extra-cellular lysosomal hydrolytic enzyme levels. These results are compatible with earlier observations on dry pellet weights [9] and, we believe, indicate that the rat cotton pellet granuloma test could be instrumental in finding non-steroidal anti-inflammatory drugs with a similar mode of action to the steroids, but without the considerable hormonal side effects [6].

MATERIALS AND METHODS

Implantation of cotton pellets. Female Wistar rats (Olac, Oxfordshire, 130–150 g) were anaesthetised with halothane (Fluothane, I.C.I.), and two sterile cotton wool pellets cut from No. 1 dental roll (Ash, Birmingham, U.K.) were implanted subcutaneously in each rat, one each side of a ventral mid-line incision. After closing the wound with a Michel clip the animals were allowed to recover. For each experiment pellets were of equal weights ± 1 mg.

Drugs were administered orally suspended in 0.7% methyl cellulose starting on the day of implantation (day 0) or at indicated times afterwards. Body weights were recorded throughout the study and at the termination of each experiment the animals were killed either by cervical dislocation when the pellets were to be used for biochemical studies, or by a lethal intraperitoneal dose of pentobarbitone (Nembutal, Abbot) when weights of pellets and organs were the only parameters measured.

Differential cell counts. Pellets were removed from rats at various times after implantation and halved with a scalpel. A smear was made from each half onto

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Table 1. The effects of steroids on granuloma and thymus weights and body weight gains

Compound	Dose mg/kg p.o.	Granuloma (mg ± S.E.M.)	Per cent inhibition of granuloma dry weight	Thymus wt. (mg)	Body wt. gain (g)
Methyl-cellulose	—	43.9 ± 3.7	—	500 ± 31	20.5 ± 1.7
Hydrocortisone	10	28.5 ± 2.1	35†	251 ± 18†	9.5 ± 0.9†
Methyl-testosterone	1	40.2 ± 3.1	8	391 ± 19†	17.5 ± 10
Ethinyl Oestradiol	0.035	40.9 ± 2.8	7	455 ± 24	11.5 ± 1.7†
Methyl-cellulose	—	55.7 ± 4.0	—	392 ± 43	18.9 ± 1.2
Dexamethasone	0.015	29.8 ± 2.6	46*	254 ± 26†	8.9 ± 0.9*

10 ♀ OLAC Wistar rats per group (original wt. range 130–150 g) dosed days 0–5, measurements made on day 6. compared using Student's group t test.
* $P < 0.001$.
† $0.001 < P < 0.01$.

microscope slides which were then air dried. The slides were stained with a modified Wright–Jenner stain using an Ames automatic staining machine. A Lynx microscope (Gillet and Siebert) was used for differential cell counts. 100 cells being counted on each slide.

Determination of granuloma formation. Pellets were removed from groups of 10 rats, placed on glass dishes and dried overnight at 80° before weighing. The mean granuloma weight per rat was calculated and used in the comparison between control and test groups, the original weight of the pellet being known. The Student's Group t test was used to evaluate the results.

Determination of wet weight, DNA content and [^{3}H]thymidine incorporation into DNA. The method used to study [^{3}H]thymidine incorporation was adapted from that used by Kulonen and Potila [11]. Pellets removed from rats at various times after implantation were teased apart in Krebs–Ringer + 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) (Sigma) at pH 7.4. Three pellets were placed in 5.0 ml of warm buffer (37°) in pre-weighed 25 ml conical flasks. After re-weighing to determine the wet weight of the pellets the flasks were incubated at 37° for 15 min in air. Then 10 μl of [6–^{3}H]thymidine (10 μCi; sp. act. 23 Ci/m-mole, Radiochemical Centre, Amersham, U.K.) was added to each flask. After a further incubation for 3 hr at 37° with constant shaking, the reaction was stopped by the addition of 10 ml ice-cold 1N perchloric acid (PCA) to each flask which was then placed in ice. The pellets and precipitated material were collected by centrifugation (600 *g* for 10 min at 4°) in polycarbonate conical-centrifuge tubes containing conical polyethylene support funnels (CSTI, Amicon, High Wycombe, U.K.). The pellets were retained in the funnels and the precipitated material collected in the tube. Both pellets and precipitate were than washed five times with 3 × 10 ml cold 0.5 N PCA and 2 × 10 ml cold 80% ethanol. The cotton pellets and sediments were then resuspended in 4 ml 0.5 N PCA and heated at 90° for 15 min to hydrolyse the DNA. After centrifugation, the supernatants were removed. Duplicate 0.2 ml samples were taken for DNA estimation by the diphenylamine method of Burton [12] and duplicate 0.2 ml samples were added to 7.0 ml of NE 260 scintillation fluid (Nuclear Enterprises, Edinburgh) for the assay of radioactivity. Counting was performed in a Packard 2450 Scintillation Spectrophotometer. Non-implanted cotton pellets were carried through the whole incubation and extraction procedure to determine the effectiveness of the washing process for [^{3}H]thymidine removal. Only background counts (< 30 c.p.m.) were found in these pellets.

DNA, protein and lysosomal enzyme determinations in pellets and exudates. Pellets were removed from the rats at various times after implantation and placed in the conical polyethylene supports held in polycarbonate centrifuge tubes and centrifuged at 1000 *g* for 10 min at 4°. The clear cell-free exudate fluid was collected in the tube, decanted from the cell pellet and used for the measurement of protein and the determination of hydrolytic enzyme levels. The contents of the cones (pellets and adhering cells) were pooled with the resuspended cell pellets in the tubes and used for DNA determination as described above.

β-Glucuronidase (β-G, EC 3.2.1.31) and β-*N*-acetylglucosaminidase (NAG, EC 3.2.1.30) were assayed in twenty-fold aqueous dilutions of pellet exudates. β-G was assayed with phenolphthalein β-D-glucosiduronic acid (6.25×10^{-4} M) as substrate in sodium acetate buffer (pH 4.5) at 37° [13]. *p*-Nitrophenyl β-D-*N*-acetyl glucosaminide (1.6 mM) was used as the substrate for NAG, in citrate (0.05 M) phosphate (0.1 M) buffer at pH 4.5 [14]. Sodium chloride (0.1 M), which activated the exudate enzyme by 20 per cent, was included in the assay mixture.

When large numbers of samples were estimated, the above assays were adapted for use on an auto-analyser system incorporating a Camlab fraction collector, Gilson pump, Camlab incubating oil bath and Cecil spectrophotometer. Calibration of the system was made with twice recrystallised *p*-nitrophenol in place of substrate. Enzyme activities were expressed as nmoles of substrate hydrolysed per hr at 37° per mg exudate protein. Exudate protein assays were done by the method of Lowry *et al.* [15].

RESULTS

Effects on dry granuloma weight. Drug activity was assessed by comparison of 6-day granuloma weights

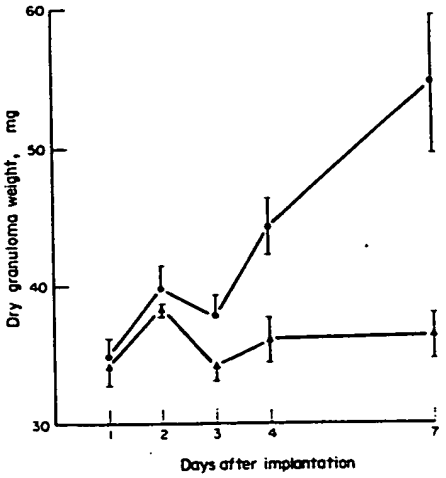

Fig. 1. Dry granuloma weights at various times after implantation. Each point represents the mean ± S.E.M. of 10 samples. Pairs of pellets were dried overnight at 80° after removal of exudate by centrifugation. ●——● Control; ▲——▲ hydrocortisone 10 mg/kg p.o.

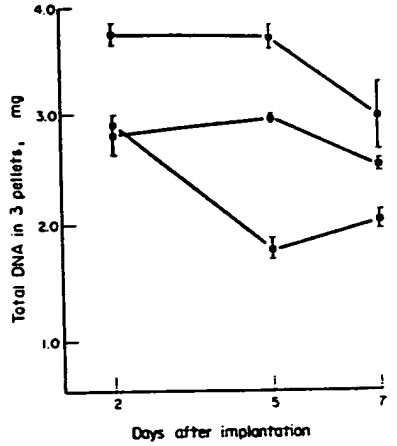

Fig. 3. Total DNA levels in control pellets. Results from three experiments, mean and range of three samples from three pellets in each case.

from groups of animals dosed from days 0–5. In Table 1 it can be seen that both hydrocortisone and dexamethasone significantly reduced dry granuloma weights, 15 μg/kg/day of the latter producing an effect equivalent to that of 10 mg/kg/day of hydrocortisone. In confirmation of previous reports [2, 3], both compounds caused a reduction in the weight of the thymus gland and reduced the body weight gain. Two other steroids without reported anti-inflammatory activity, methyl testosterone and ethinyl oestradiol, did not reduce dry granuloma weights when dosed on the same schedule.

When pellets were taken at different times after implantation, the effect of hydrocortisone on dry-weight was apparent on the third day (10 per cent reduction in dry weight, Fig. 1) and coincided with an observed hydrocortisone-induced inhibition of body weight increase. It should be noted that granuloma dry weight in Fig. 1 is that in pellets from which the exudate has been separated.

Differential cell counts and DNA content of granulomas. Differential white cell counts from 2, 5 and 7 day pellets are shown in Fig. 2. At day 2 the population was predominantly polymorphonuclear leucocytes (PMNL) (> 70 per cent) with smaller numbers of macrophages and lymphocytes. The overall population is thus typically that found at the site of an acute inflammatory response. At 5 and 7 days the percentage of PMNL cells fell and a concomitant increase in the macrophage and lymphocyte populations was observed. This change in cell population indicated that the inflammatory reaction had progressed from one of an acute to a chronic nature. To show that the population changes were real and not a function of cell recovery from the pellets, DNA content in the pellets was measured and the results from 3 experiments are shown in Fig. 3. Generally DNA content at day 2 was as high as at day 5 and 7 and it is possible therefore that a cell population was quickly established at the site of inflammation and that cell types changed within the population.

[^{3}H]*thymidine incorporation.* When DNA synthesis was examined by uptake of [^{3}H]thymidine, it was found that although DNA levels in the pellets were equally high on days 2 and 5 (Fig. 3), no DNA synthesis was found until the 5th day (Fig. 4a). Further experiments (Fig. 4b) showed that synthesis commenced between the third and fourth day. These results were compatible with the observed influx of non-proliferating PMNL cells immediately after pellet implantation (Fig. 2), and the population then being replaced by proliferative

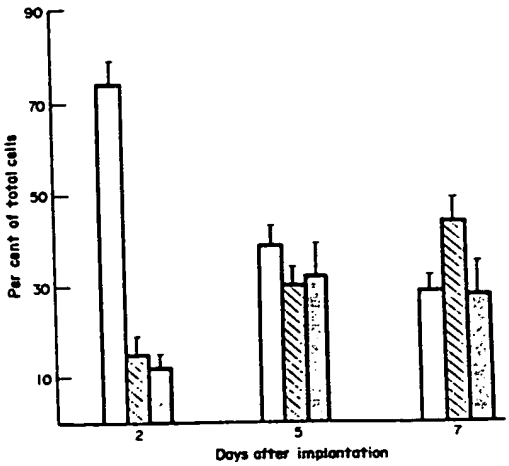

Fig. 2. Differential cell counts from cotton pellets. Each column represents the mean ± S.E.M. of 8 determinations, 100 cells counted on each slide.

▨ PMNLs: ▧ lymphocytes: □ monocytes/macrophages.

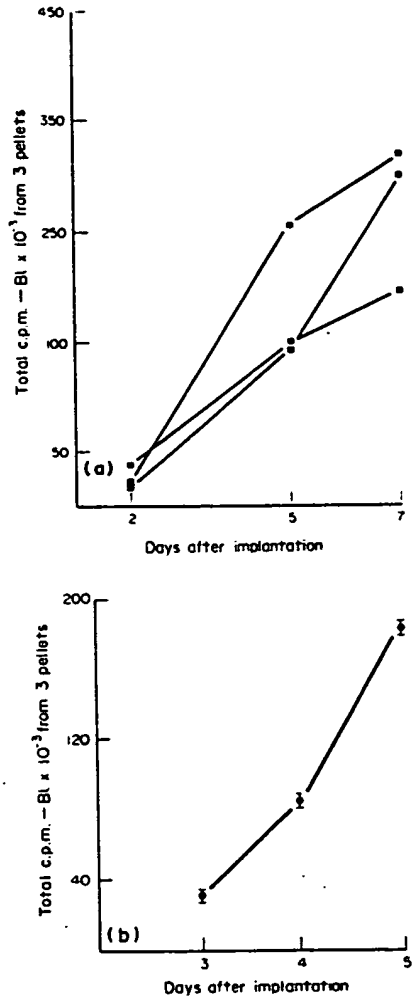

Fig. 4. [^{3}H]thymidine incorporation *in vitro* in cotton pellet induced granulomas. (a) Results from three experiments, means of three samples from three pellets in each case. (b) Each point represents the mean ± S.E.M. of 6 samples from 3 pellets each.

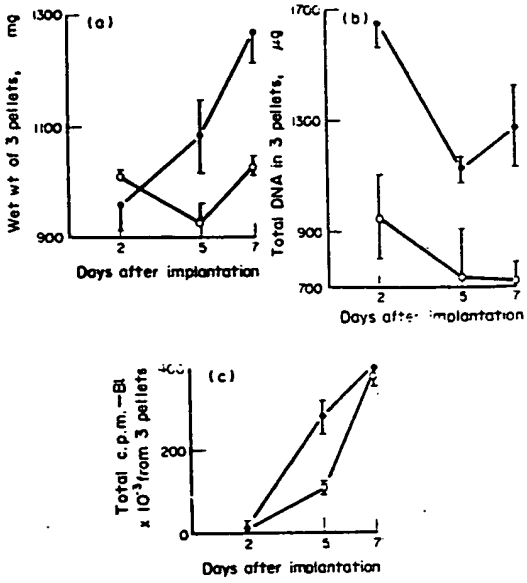

Fig. 5. The effects of hydrocortisone on wet pellet weight, total DNA levels and [^{3}H]thymidine incorporation *in vitro*. Each point represents the mean ± S.E.M. of 3 samples from 3 pellets each. ●——● Control; ○——○ hydrocortisone 10 mg/kg/day p.o.

mononuclear cells. It is possible that some of the observed DNA synthesis is due to the presence of fibroblasts and this is under investigation.

Effect of hydrocortisone on granuloma development. Hydrocortisone (10 mg/kg) reduced the wet weight of pellets on day 2, day 5 and day 7 (Fig. 5) in animals dosed days 0–1, 0–4, 3–6 respectively. At all times, the cellular content of the pellets (from the DNA levels) was significantly reduced (Fig. 5), suggesting that infiltration of both PMNL in the acute phase and monocytes in the chronic phase had been prevented.

It is worth noting that, in contrast to Fig. 3, in the period between the loss of PMNL and influx of monocytes (between 2 and 5 days) the cell content of the pellet was reduced. This phenomenon, although variable, was frequently observed.

[^{3}H]thymidine incorporation. Uptake of [^{3}H]thymidine (DNA synthesis) was significantly depressed only at 5 days by hydrocortisone (Fig. 5). This effect may be interpreted as a delay in the onset of DNA synthesis, rather than direct inhibition of DNA synthesis, as at 7 days the drug-treated group and the controls had the same levels of [^{3}H]thymidine uptake in the pellets.

Soluble acid hydrolase levels in pellet exudates. In addition to the studies on the development of the cotton pellet granuloma itself, exudates from developing granulomas were collected and assayed for levels of acid hydrolases known to be present in both PMNL and macrophages [16]. The levels of enzyme activity found in exudates were compared with those in plasma from normal rats (Table 2). When expressed as enzyme activity per ml of fluid, exudates at both 2 and 4 days had NAG and β-G activities 10 to 100-fold greater than those in rat plasma. The difference was even more pronounced when specific activities were compared.

The lower enzyme levels at 4 days (cf. 2 days) were almost entirely accounted for by the lower protein levels at this time (Table 2); the specific activities were reduced by only 15 per cent (NAG) and 22 per cent ($P < 0.05$) (β-glucuronidase).

Effect of hydrocortisone on soluble enzyme levels. Groups of rats were dosed with hydrocortisone at 10 mg/kg/day p.o. Exudate enzyme levels were then determined at days 1, 2, 3, 4 and 7 (Fig. 6a and b). Compared with the untreated pellet implanted controls, the exudate enzyme specific activities were reduced at all times by hydrocortisone and in substantially the same way for both NAG and β-G (Fig. 6a and b respectively). The time of lowest activity at 3 days

Table 2. Activities of enzymes in cotton pellet exudates

	Day	Protein (mg/ml)	Activity (nmoles substrate hydrolysed/hr. at 37°) NAG Activity/ml ±S.E.M.	NAG Specific activity ±S.E.M.	β-G Activity/ml ±S.E.M.	β-G Specific activity ±S.E.M.
Plasma	0	83 ±2.7	556 ±38	6.7 ±0.46	14.2 ±0.16	0.17 ±0.02
Pellet Exudate	2	66 ±1.4	6052 ±422	91.7 ±6.8	1432 ±104	21.7 ±1.4
Pellet Exudate	4	54.9 ±1.1	4312 ±384	78.5 ±7.2	934 ±88	17.0 ±1.6

10 ♀ OLAC Wistars per group, original weight range 130–150 g.

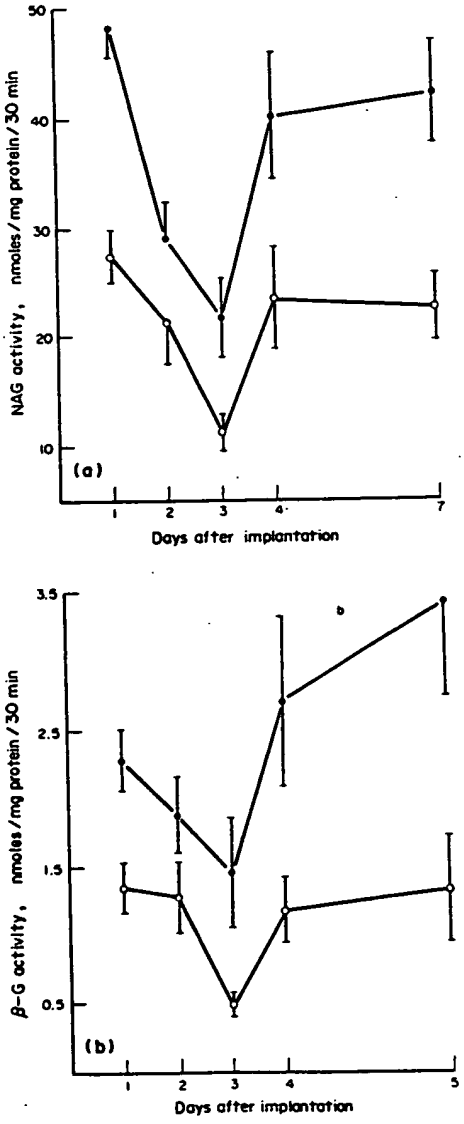

Fig. 6. The effect of hydrocortisone on soluble hydrolytic enzyme activity in cotton pellet exudates.
(a) NAG activity. (b) β-G activity.
●——● Control. O——O Hydrocortisone 10 mg/kg/day p.o.
Each point represents the mean ± S.E.M. of 10 samples of exudate, each from 2 pellets.

coincides with the presumed loss of PMNL and initiation of the influx of monocytes (Fig. 2). On the fourth and seventh days the trends show the effect of the steroid to be more marked, having prevented any significant increase in hydrolase activity for either enzyme over the period (Fig. 6a and b). The same effect was observed on the pellet dry weight when a significant increase in the controls ($P < 0.05$) was totally suppressed in hydrocortisone-treated animals (Fig. 1).

DISCUSSION

The present experiments have demonstrated some features of the inflammatory response to implantation of cotton pellets in rats and the effects of steroids.

During the first 2–3 days PMNL cells were predominant and DNA synthesis could not be detected. As measured by DNA content at this time, the accumulation of these cells was depressed by hydrocortisone. In agreement with these findings, Davies and Thompson [17] demonstrated that, in mice, the steroid paramethasone inhibited PMNL infiltration into peritoneal exudates after injection of thioglycollate medium.

From 2–7 days after cell implantation DNA levels in control pellets rose slightly and were again depressed in pellets from steroid-treated rats. However, the effect of the steroid on DNA synthesis (i.e. cellular proliferation) was to delay its onset, rather than depress it. Thus at 7 days, after treatment restricted to the proliferative phase of the response, the rate of DNA synthesis was not significantly different from that of the controls. As the total cell content was reduced by this dosing schedule, we conclude that hydrocortisone inhibits cellular infiltration during both phases of the pellet response. During the proliferative phase, therefore, after steroid treatment a greater proportion of the cells are undergoing cell division either because fewer non-proliferative cells enter the pellet or, more likely, more cells are induced to divide, to compensate for the lower number of macrophages at the inflammatory site. The effect of hydrocortisone on soluble acid hydrolase levels supports this concept, since although DNA content rose between days 5 and 7, the increase in soluble enzyme activity between these times was almost completely abolished by the drug.

In control pellets at 2 days death and autolysis of the accumulated PMNL (rich in lysosomal enzymes) would be expected to result in a substantial release of acid hydrolases. The observed high levels of NAG and β-G compared with plasma activity at 2 days support this hypothesis, especially as soluble activity had reached a minimum by 3 days, when the granulocytes would be starting to give way to proliferative mono-

cytes (macrophages). With the advent of monocytes into the pellet a second rise in acid hydrolase activity may have reflected the release of lysosomal enzymes during phagocytosis of debris in the inflamed area.

The biochemical separation of the granuloma reaction into two phases, corresponding to the PMNL and monocyte functions, makes it possible to study each phase independently. Using the DNA assay described and administering the drug for 2 days, compounds which will inhibit PMNL infiltration and thus control the acute phase of an inflammatory response can quickly and easily be detected. Conversely, longer dosing schedules coupled with investigations of cell type, DNA levels and biochemical parameters can be used both to elucidate mechanisms of the proliferative phase response and subsequent changes involving the accumulation of fibroblasts and the laying down of collagen. Tests based on the later stages of the granuloma response should help in identifying "steroid-like" drugs, having specific effects on selected parameters e.g. pellet dry weight, granuloma collagen content and macrophage activity.

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